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Inactivation of aerosolized *Bacillus anthracis* surrogate spores in close proximity to the flame: Simulation study

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ABSTRACT

Survival of bio-warfare aerosol agents exposed to different environmental conditions has gained substantial attention in the biodefense and biosecurity research communities. The present simulation study aimed at investigating the survival of aerosolized bacterial spores in close proximity to a flame under test conditions relevant to a fire or explosion in a bio-weapon facility. The tests were conducted with aerosolized spores of *Bacillus thuringiensis var. kurstaki* (Btk), a well-recognized surrogate of *Bacillus anthracis*, which is a common bio-warfare agent. The loss in spore viability resulting from their exposure to a peripheral area of an air-acetylene flame at two particle-to-flame proximity levels was experimentally determined for different exposure time intervals ranging approximately from 0.1 to 6.0 s. The spore inactivation was quantified through comparison of the viability levels obtained for exposed and non-exposed (control) samples. At relatively short time intervals, the inactivation rate was close to exponential. However, as the exposure time increased, the spore viability decrease slowed down for both proximity levels. The "breaking point" was found to be dependent on the particle-to-flame proximity. The findings point to a small fraction of spores that exhibits particularly high resistance to the stress from the flame-originated heat. To verify the existence of such a sub-population, a separate experiment was performed, in which the aerosolized spores that survived the stress caused by exposure to flame were subsequently subjected to an additional (post-exposure) stress by being placed in an oven for 5, 10 and 20 s. The study results demonstrated the presence of "super-resistant" Btk spores. This justifies the need of developing special materials with pronounced biocidal capabilities (well in excess of those associated with an air-acetylene flame) in order to effectively inactivate "super-resistant" aerosolized bio-agents.

1. Introduction

It has been recognized that many bacterial species (including *Bacillus*) are capable of forming dormant cellular structures, defined as spores, for surviving harsh environmental conditions (Grinshpun et al., 2012, 2017; Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000). Most bacterial spores are close to 1 μm in aerodynamic diameter; they can be easily dispersed and remain airborne for a long time (Grinshpun et al., 2010; Li, 1999). Furthermore, aerosolized spores may maintain their initial viability while being exposed to natural ultraviolet radiation and other atmospheric factors. This is fully relevant to *Bacillus anthracis* spores that have received considerable attention because of the association with biological warfare and bioterrorism (Inglesby et al., 2002; Turnbull,

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2002). In an event where a bio-weapon facility storing *B. anthracis* is targeted by military means, these highly pathogenic microorganisms, once aerosolized, can survive the explosion and/or fire in the facility and be subjected to the short- or long-range atmospheric transport. The latter presents an enormous threat to large populated areas that may be located hundreds or thousands of kilometers from the source (Nelson, 2004).

There have been numerous studies that aimed at developing effective inactivating methods/products against aerosolized bioagents such as, wet and dry heat, oxidizers, germicidal ultraviolet and microwave irradiation, and certain biocidal chemicals [e.g., (Brickner et al., 2003; Burton, Adhikari, Iossifova, Grinshpun, & Reponen, 2008; Lin & Li, 2002; Rogers et al., 2007; Rogers, Choi, Richter, Stone, & Taylor, 2008; Setlow, 2006; Wu, SyBing, Wang, & Zachariah, 2017; Wu & Yao, 2010; Zhang et al., 2010; Zhou, Orr, Lee, & Zachariah, 2016; Zhou et al., 2015)]. Some recent efforts utilized iodine embedded into reactive powdered materials. The iodine released into the flame during combustion of such a powder can efficiently inactivate airborne spores [e.g., (Aly et al., 2014; Clark & Pantoya, 2010; Farley & Pantoya, 2010; Grinshpun et al., 2017; Grinshpun et al., 2010; Nakpan et al., 2018; Sullivan et al., 2010; Wang, Corcoran, Leybova, & Dreizin, 2015; Wang et al., 2015; Zhang, Schoenitz, & Dreizin, 2010)]. Notably, there is a lack of data in peer-reviewed literature on the spore inactivation in close proximity to a regular flame.

This simulation study aimed at generating the spore inactivation database using *Bacillus thuringiensis var. kurstaki* (Btk) spores as a surrogate for *B. anthracis* (Greenberg, Busch, Keim, & Wagner, 2010; Tufts, Calfee, Lee, & Ryan, 2014). The laboratory tests involved two spore-to-flame proximity levels. The loss in spore viability resulting from their exposure to a peripheral area of an air-acetylene flame was quantified as a function of exposure time that ranged approximately from 0.1 to 6 s. The study findings pointed to a small fraction of spores that was particularly highly resistant to the stress from the flame-originated heat. A separate experiment has proven the existence of such “super-resistant” bioaerosol fraction.

2. Materials and methods

2.1. Experimental setup and conditions

This investigation adopted the state-of-the-art laboratory facility developed and built at the University of Cincinnati and used in several studies (Aly et al., 2014; Grinshpun et al., 2012, 2017; Grinshpun et al., 2010; Nakpan et al., 2018). The experimental setup was operated inside a Class II biological safety cabinet (BSC) (Model 6TX, Baker Co., Inc., Sanford, ME, USA). The main element of the setup was a cylindrical exposure chamber in which the airborne spores were exposed at a specific distance to the flame.

The Btk suspension prepared by suspending dry Btk spores in sterile deionized water was aerosolized from a six-jet Collison nebulizer (BGI Inc., Waltham, MA, USA). Operating at a flow rate of 6 L min^{-1}, this nebulizer introduces a strong shear force at the dispersion point that helps efficiently de-agglomerate the spores during their aerosolization. Prior to each experiment, the nebulizer operated for 5–10 min to achieve and maintain a constant bioaerosol concentration in the chamber. Mixing the bioaerosol generated from the suspension with HEPA-filtered dry air led to shrinking of the nebulizer-generated particles to the actual spore size as the water evaporated rapidly (Grinshpun et al., 2010). The air flow used for dilution had a flow rate of 6, 12, 30, or 48 L min^{-1}. The total air flow and the length of the exposure chamber were variables that allowed us to conduct the tests at different exposure time intervals: approximately 0.1, 0.3, 0.6, 1.2, 2.0, 4.0, or 6.0 s (calculated based on the average flow velocity).

The challenge bioaerosol passed through a 10-mCi ^{85}Kr particle charge equilibrator (TSI, Shoreview, MN, USA) and then entered the exposure chamber where the particle size distribution was measured using an optical particle size spectrometer (Grimm Model 1.108, Grimm Technologies, Inc., Douglasville, GA, USA). This measurement confirmed that the particle population was mostly represented by single spores with a very small fraction of agglomerates. It is evident from the peak of the particle size distribution of *Bacillus* endospores generated by the Collison nebulizer in the exposure chamber: approximately 0.71 μm (Grinshpun et al., 2010). Once they entered the exposure chamber, the bioaerosol particles passed above the air-acetylene flame. The flame type was chosen because the combustion products of acetylene produce no or little biocidal effect on aerosolized *Bacillus* spores (Aly et al., 2014; Grinshpun et al., 2017). The flame was ignited from the burner, which position is adjustable to create different spore-to-flame proximity levels and, consequently, different temperature profiles in the exposure chamber. A specially designed set of electrical heaters installed along the chamber allowed maintaining a uniform longitudinal temperature profile. Five thermocouples (Omega Engineering, type E, part # TJ36-CAXL-116E-6, Norwalk, CT, USA) were used to monitor the temperature in the exposure chamber.

After passing a 300-mm long cooling system installed at the exit of the chamber, the spores were collected on three identical sterile 25-mm diameter gelatin filters placed into sampling cassettes (SKC Inc., Eighty-Four, PA, USA). It is noted that using gelatin filters could have caused some desiccation-driven loss of viability for sensitive vegetative cells (Yao & Mainelis, 2006), but not for the *Bacillus* spores tested in this study which are known to be highly resistant to desiccation (Nicholson et al., 2000). Thus, the bioaerosol collection process did not introduce any systematic error. The sampling flow rate was set at 5 L min^{-1} for each cassette.

The experiments were conducted with the burner adjusted at two proximity levels, measuring from the burner tip to the central axis of the exposure chamber. Accounting for the chamber inner diameter of 50 mm, the spore-to-flame proximities tested in this study were 375 ± 25 mm and 415 ± 25 mm. The temperature profiles in the exposure chamber were quantified cross-sectionally and longitudinally to determine the pronounced spatial non-uniformity of the air temperature. At these two distances, the weighted average temperatures $< T_{air} >$ were approximately 260 °C (± 25 °C) and 170 °C (± 15 °C), respectively.

The collected samples were analyzed using the conventional culture-based method to determine the concentration of viable spores that survived the exposure to stress and compare it to the controls (concentration of viable non-exposed spores). It is acknowledged that this study was focuses solely on culturable spores, and the culture-based method may underestimate the number of viable spores compared to other techniques such as DNA stain method (Li, Mendis, Trigui, Oliver, & Faucher, 2014). The

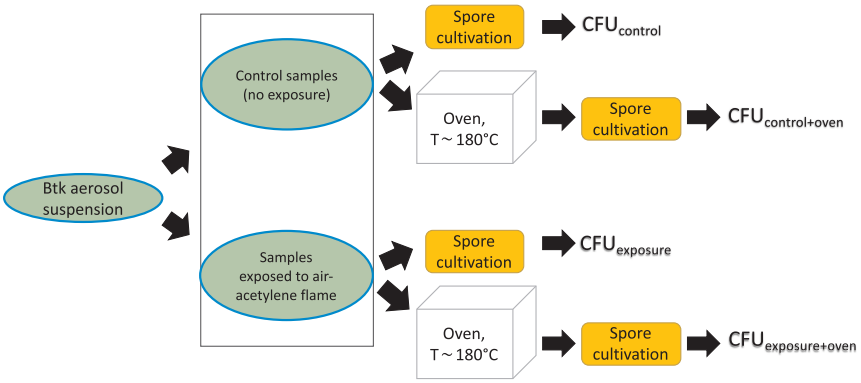

Fig. 1. Experimental protocol (schematics).

concentrations were expressed in colony forming units per cubic meter of the sampled air (CFU/m^3). For control samples, the experiment was repeated with neither the burner nor the heaters operating. In each experiment, at least four controls were generated with the spore aerosol concentration being approximately 10^7 CFU/m^3. The inactivation factor (*IF*) was calculated as follows:

$$IF = \frac{CFU_{control}}{CFU_{exposure}}$$

where $CFU_{control}$ is the total number of viable spores collected in the control samples, and $CFU_{exposed}$ is the number of viable spores collected in the exposed sample.

2.2. Post-exposure study

As presented in the Results and Discussion section below, the results of inactivation experiments pointed to a sub-population of spores that appeared to exhibit particularly high resistance to the flame-generated heat. To definitively prove this finding, a separate experiment was conducted to introduce an additional (post-exposure) thermal stress to the bio-agents. Spores of Btk were first exposed to the thermal stress generated by an air-acetylene flame in a similar manner described in the above sub-section ("Experimental setup conditions"). The exposure temperature and the exposure time were varied to achieve different *IF* values. For each experiment, twelve exposed samples were collected. Six were randomly selected for cultivation immediately after the thermal exposure and defined as $CFU_{exposure}$. Subsequently, another six filters were placed in an oven (Isothemp oven, Model 737F, The Fisher Scientific, Dubuque, Iowa) to be exposed to additional stress and subjected to cultivation (see Fig. 1) named as $CFU_{exposure+control}$. The temperature in the oven was set at 180 °C (± 5 °C). The only variable in this phase of the study was the time in the oven, which was 5, 10, or 20 s. The post-exposure inactivation ratio was determined as:

$$Post - exposure\ inactivation\ ratio = \frac{CFU_{exposure}}{CFU_{exposure+oven}}$$

where $CFU_{exposure}$ is the number of viable spores collected in the exposure chamber test with the burner "on" and $CFU_{exposure+oven}$ is the residual number of viable spores that survived both the flame-imposed stress in the chamber and the additional thermal stress from the oven. We note that since gelatin collection filters could not keep their integrity under the oven conditions, they were replaced in this post-exposure study with polytetrafluoroethylene (PTFE) membrane filters (MilliporeSigma, Burlington, MA, USA).

2.3. Preparation of challenge bioaerosol

B. thuringiensis serovar kurstaki, Btk, strain SA-11 (product # SA-11 SDTC; technical grade concentrate developed for the US Army and Air Force) was used for suspension preparation. Additional details regarding the preparation of microbial suspension have been described in our recent publications (Grinshpun et al., 2017; Nakpan et al., 2018). To sum up, crystal proteins (insecticidal) were removed from freeze-dried Btk spores purchased from Certis USA Inc. (Columbia, MD, USA) through a purification process. As the next step, the spores were repeatedly washed by vortexing and centrifugation at room temperature until the supernatant was clear and re-suspended in sterile filtered deionized water. Before use in experiments, the suspension was vortexed and sonicated for 5 min to reduce the spore agglomeration. The suspension had a culturable spore concentration of ≈ 10^8 –10^9 CFU mL^{-1}.

2.4. Analysis of the collected microbial samples

After the samples were collected, spores were extracted immediately from the membrane filters by immersing them into 5 mL of sterile filtered deionized water and vortexed for 2 min. The aliquots varying from 100 μL to 5 mL were cultivated on tryptic-soy agar (TSA, BD, Franklin Lakes, NJ, USA) in three identical plates by incubating at 37 °C for 24 h (Adhikari et al., 2016; Aly et al., 2014;

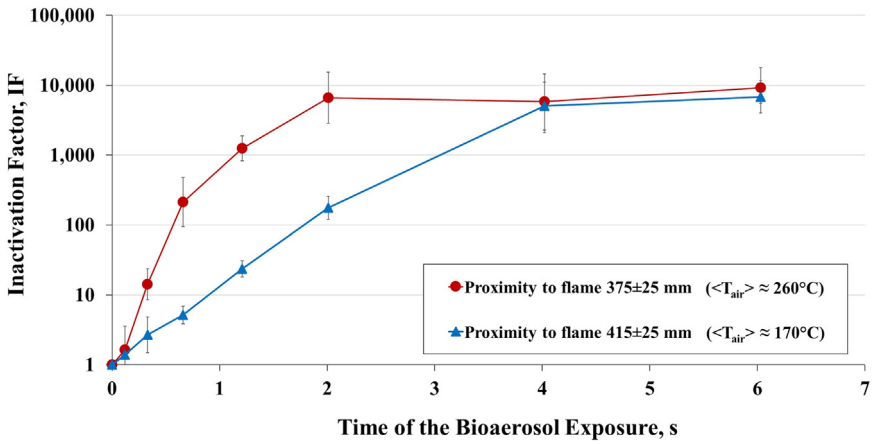

Fig. 2. Effect of time of the bioaerosol exposure on the inactivation of Btk spores. Each point represents a geometric mean and the bar represents the geometric standard deviation calculated from 4 to 12 replicate measurements.

Grinshpun et al., 2012, 2017; Nakpan et al., 2018). The CFU enumeration was performed immediately after the 24-h incubation.

2.5. Data analysis

It was determined that a log-normal distribution fit the data best. Therefore, the geometric mean (GM) and the geometric standard deviation (GSD) of the *IF* values were calculated for each set of replicates. The spore inactivation produced by different conditions were compared using an analysis of variance (ANOVA). Segmented regression models were applied to verify the data sets. A p-value of ≤ 0.05 was regarded as statistically significant.

3. Results and discussion

3.1. Inactivation of Btk bioaerosol at two spore-to-flame proximity levels

Fig. 2 presents the relationship between *IF* and the time of bioaerosol exposure obtained for the two tested spore-to-flame distances. The relationships obtained at different spore-to-flame proximity levels are statistically different ($p < 0.05$).

For the greater proximity (415 ± 25 mm), the *IF* exponentially increased as a function of the exposure time until the latter reached approximately 4 s. However, further increase of the duration did not enhance the spore inactivation, and *IF* remained constant between 4 and 6 s. It is seen from Fig. 2 that the inactivation plateau occurred at *IF* of about 9000, which is translated to the spore survival of 0.011%.

As expected, for the closer proximity (375 ± 25 mm) that generates greater heat stress, $< T_{air} > 260$ °C, *IF* increased more rapidly (Fig. 2). Initially, at a relatively short exposure time from approximately 0.1 to 0.6 s, the inactivation exponentially increased with the time, but then slowed down and stopped increasing at about 2 s. The plateau level was essentially the same: $IF \approx 9000 – 10{,}000$.

Since our experimental facility was designed to allow measuring very high inactivation levels, significantly in excess of 10,000 (Aly et al., 2014; Grinshpun et al., 2017; Nakpan et al., 2018), the plateau obtained in this study does not represent the limit of detection of our test protocol. Instead, the results suggest that some bacterial spores did not lose their viability with the increase of exposure time.

The common factors that are responsible for resistance of a *Bacillus* spore include high core Ca^{2+} levels, low core water content, and DNA binding with α/β-type small acid-soluble spore proteins (SASPs) (Nicholson et al., 2000; Setlow, 2006). For the dry heat inactivation, the viability loss for *Bacillus* endospores has been associated with DNA mutational damage (Johansson, Adhikari, Reponen, Yermakov, & Grinshpun, 2011; Setlow et al., 2014; Setlow, 2006). The α/β-type SASPs would contribute to the spore resistance in a major way (Fairhead, Setlow, & Setlow, 1993). It is recognized that after the bioaerosol exposure to heat, the structure of the survived spores could have changed due to increased number of DNA bindings with the α/β-type SASPs (Russell, 2003). The role of α/β-type SASPs has been validated in earlier studies; the investigators reported that these SASPs can protect the DNA from depurination or inhibit the process of depurination, thus increasing the resistance of *Bacillus* spores to dry heat stress (Fairhead et al., 1993; Setlow & Setlow, 1995). This helps explain the development of the fraction of “super-resistant” spores identified in our experiment, which survived regardless of the bioaerosol exposure duration, at least up to about 6 s. Furthermore, our finding is consistent with the report of Movahedi and Waites (2000) who asserted that *Bacillus* spores, which were subjected to pre-heat treatment, subsequently developed an elevated resistance to heat. Similar results in enhancing the heat resistance were observed when *Bacillus* spores were pre-exposed to acid shock treatment (Lee, Movahedi, Harding, & Waites, 2003). Overall, exposing spores to dry heat can help develop an appreciable heterogeneity, and the latter allows for a sub-population of spores capable of withstanding

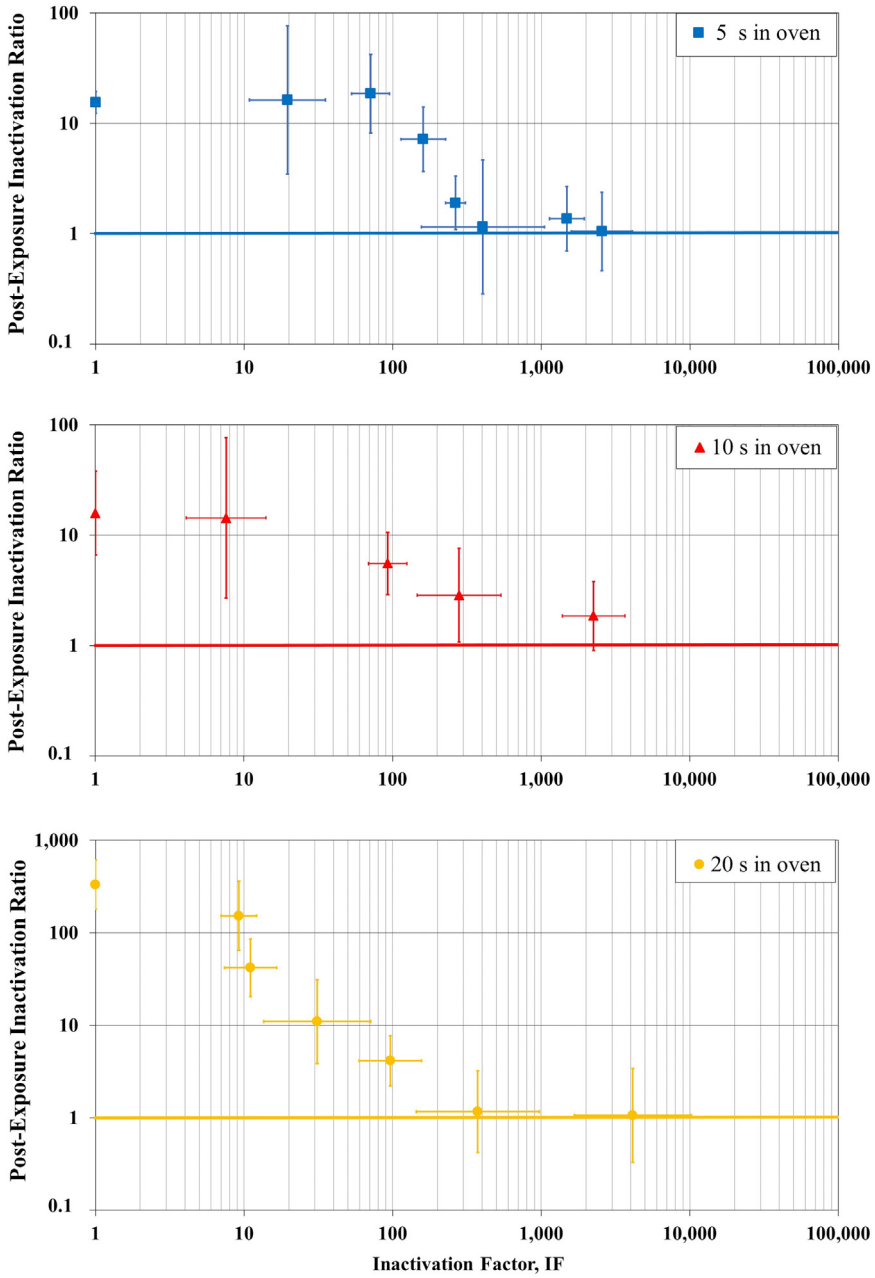

Fig. 3. Relationship between the *IF* due to bioaerosol exposure and the post-exposure inactivation ratio for aerosolized Btk spores at three time intervals of the oven treatment: (A) 5 s, (B) 10 s, and (C) 20 s. Each data point represents a geometric mean and the bar represents the geometric standard deviation calculated from 6 replicate measurements.

greater heat stress than the "regular" ones (Pandey & Brul, 2016; Russell, 2003).

3.2. Post-exposure inactivation ratio

A separate experiment, which was conducted to verify the existence of the above-mentioned "super-resistant" Btk spores through introducing an additional (post-exposure) thermal stress, generated results that are presented in Fig. 3. Here the post-exposure inactivation ratio is plotted against the *IF* obtained from testing the bioaerosol exposure in the chamber. Different *IF* values reflect different levels of stress imposed on the aerosolized spores in close proximity to the flame. The 5-s placement to the oven (Fig. 3A) produced a post-exposure inactivation ratio of about 15, which translates to the survival of approximately 7% of spores in the oven. One would expect that the effect of heating in the oven would not depend on the prior bioaerosol exposure. However, at $IF \approx 100$ the

post-exposure inactivation ratio began decreasing with the *IF* increase, eventually plateauing at 1 as *IF* continue increasing. This suggests that the spores, which survived particularly high stress during their near-flame exposure, gained additional resistance and thus were not "killed" by the 5-s oven treatment. Similar results were obtained for the 10- and 20-s oven treatments (Figs. 3B and C). For the 10-s in the oven, the relationship between the post-exposure inactivation ratio and *IF* was not significantly different from that obtained for the 5-s in the oven ($p > 0.05$). The post-exposure inactivation curves for 5- and 10-s treatments started at almost the same point, and both demonstrated a decrease with a plateau trend at higher *IF* values. Noticeably high post-exposure inactivation ratios were observed for 20-s treatment in the oven when $IF \leq 10$, yet the ratios dropped and became a plateau once the *IF* value was ≥ 300 (Fig. 3C). The findings confirm that the inactivation provided by the thermal stress via the oven became less effective for spores that gained extra resistance through their previous exposure to the flame in the aerosol phase.

It is anticipated that the negligible response of "super-resistant" spores to the oven treatment, which we identified for 5–20 s (plateau), has limitations. In other words, further increase of the oven treatment duration (beyond 20 s) will eventually lead to inactivation of all spores subjected to this treatment. For instance, Tanner and Dack (1922) reported that *Bacillus* spores were completely inactivated by dry heat treatment in 300 s at an oven temperature of 180 °C. Overall, the data presented in this section confirmed that the near-flame exposure can develop a particularly resistant sub-population of Btk spores that are capable of surviving post-exposure thermal stress.

4. Conclusions

The survival of airborne bacterial spores in close proximity to a flame was studied. The experimental design aimed at simulating a scenario that may occur as a result of fire or explosion in a bio-warfare facility. The aerosolized viable Btk spores (a well-established surrogate of *B. anthracis*) were exposed in the vicinity of an air-acetylene flame, and their inactivation was quantified at two spore-to-flame proximity levels and different exposure duration. It was shown that a small fraction of the aerosolized Btk spores might possess relatively high resistance to the flame-generated thermal stress. These "super-resistant" viable spores may not be neutralized while in close proximity to the flame and, furthermore, may develop the capability to survive substantial post-exposure thermal stresses. The latter was demonstrated in a separate experiment, in which the spores that survived the stress caused by exposure to flame were subsequently subjected to an additional (post-exposure) stress by being placed in an oven for 5, 10 and 20 s. The findings of this study suggest that the survival ability of bacterial agents in close proximity to a fireball may be underestimated by ignoring the sub-population of "super-resistant" spores. Consequently, the results emphasize the need in novel materials with added biocidal properties that can be released in the atmosphere during combustion or explosion on a bio-weapon facility in order to effectively inactivate "super-resistant" aerosolized bio-agents over relatively short time intervals.

It is noted that this study utilized the conventional culture-based method for quantifying the spore inactivation. Deployment of additional methods such as DNA stain or scanning electron microscopy could help identify and specify the spore internal damages or changes in spore morphology. These methods can be considered for the utilization in future research.

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